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Immunopurification of Bovine Placental Lactogen

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IMMUNOPURIFICATION OF BOVINE PLACENTAL LACTOGEN

By

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B.S. University of Agriculture and Forestry in Viet Nam, 1995

A THESIS

Submitted in Partial Fulfillment of the

Requirements for the Degree of

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By Dong Thi Nguyen-Bresinsky

Thesis Advisor: Dr. Charles R. Wallace

An Abstract of the Thesis Presented
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A new technique has been developed for the purification of bovine placental lactogen (bPL) from an ammonium sulfate preparation, using an immunomagnetic separation process. This alternative procedure is used in cell and molecular biology to separate various types of proteins, cells and nucleic acids due to its advantages over the traditional methods. The first step of the bPL purification system was to isolate antibody (Ab) against bPL, called sheep anti-rabbit antibody. A two milliliter column of Immobilized Protein G, elution buffer (pH 2.8) and binding buffer (pH 5.0) were used during Ab purification process. The Ab activity was monitored by using a radioimmunoassay (RIA) prior to coupling to a biomag, which consists of a suspension of magnetic particles coated with iron oxide to provide primarily amine groups. These groups allow for covalent attaching to proteins or ligands with maintaining the biological activity. A suspension of magnetic iron particles conjugated with sheep anti-rabbit IgG was used in the experiment. The coupling efficiency was 80% determined by measuring protein concentration at 280nm. The next step was purification of endogenous bPL from bovine placental homogenate, which had been prepared by an ammonium sulfate (A.S.)

precipitation. One gram of dried A.S. dissolved in 40 ml Tris HCl buffer (10mM pH 7.5) was centrifuged at 10,000 x g for 30 min. The supernatant was collected and mixed with 5 ml biomag. The particles were recovered with a magnet and the supernatant was discarded. This was followed by washing two times with Tris buffer (10mM pH 7.5). Finally, bPL was eluted from the biomag by adding 10 ml glycine (0.1M pH 3.2). Sucrose was then added to the glycine mixture to prevent protein aggregation and stored in the refrigerator for analysis. At each step of the purification process, the protein content of the sample was estimated by measuring the absorbance of light at 280 nanometers with a spectrophotometer. The Bradford assay was also utilized for further examining of protein level in the sample. The presence of bPL in the sample was determined by RIA. The purified bPL fraction was concentrated by lyophilization and run on the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to determine how pure the bPL is. The purity level of the bPL was 25%, which is higher than results obtained in some previous studies using a similar starting material preparation. This result, however, is still not much greater than obtained with previous conventional bPL purification approaches, and other studies using the immunomagnetic separation techniques to isolate the target biomolecules. Thus further purification steps are required. The purification system described in this study, at present, cannot offer an efficient protocol for the isolation of bPL. The developed method, nevertheless, is simple and rapid as only a one-purification step is involved. This approach has also reached a moderate sensitivity, which may perhaps be refined to enhance the optimization. Additional investigations, regarding the conditions of elution buffers and the size of magnetic particles, may need to be taken into consideration to obtain a better result.

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LITERATURE REVIEW

Introduction

Placentas of primates, rodents and ruminants secrete one or more polypeptide hormones, including placental lactogen (PL), prolactin-related proteins (PRPs) and placental growth hormone V (GH-V). Among these hormones, PL has been investigated most extensively. The term placental lactogenic hormone was originally used, as this hormone exhibits prolactin-like (lactogenic) responses in several radioreceptor and bioassays (Anthony et al., 1995a). This placental hormone also displays a number of somatogenic activities, thus it is also called chorionic somatotropin (Byatt et al., 1992a). In the bovine placenta, researchers have discovered six PRPs, which are structurally similar to each other but distinct from the structure of bovine (b) PL (Schuller et al., 1991). There is no indication that these placental proteins are present in horses, pigs and rabbits (Anthony et al., 1995a). The placenta of these species probably lacks trophoblast binucleate cells (BCs), which are responsible for synthesizing PL.

Placental lactogen, growth hormone (GH) and prolactin (PRL) belong to a family of polypeptide hormones, which are structurally related and share several biological features. The primary structure of ruminant PLs has been characterized in cattle (Schuller et al., 1988), sheep (Chan et al., 1976; Hurley et al., 1977; Warren et al., 1990a) and goats (Sakal et al., 1998). The amino acid (AA) sequence of ovine (o) PL was 67% identical with bPL (Warren et al., 1990a), while the similarity between oPL and caprine (c) PL was higher (85%) (Sakal et al., 1998). These data indicated ruminant PLs are structurally more similar to PRL than to GH in the nucleotide and AA sequence, whereas human (h)

PL has a structure more related to hGH (Bewley et al., 1972) than to PRL (Shome and Parlow, 1977). These have contributed to the suggestion that the gene for rodent and ruminant PLs evolved from the prolactin gene family (Anthony et al., 1995b; Hurley et al., 1977).

Structure

The presence of PL throughout pregnancy has been described in the human, monkey (Kaplan and Grumbach, 1964), goat (Buttle et al., 1972), sheep (Martal and Djiane, 1975) and cow (Buttle and Forsyth, 1976). The purification of bPL was initially reported with the molecular weight (MW) of 22,150 and isoelectric point (pI) of 5.9 (Bolander and Fellows, 1976). However, other laboratories have not been able to repeat these findings. To clarify the situation, bPL purification and characterization were reported by a number of studies, reporting the MW of bPL ranges from 30,000 to 32,000 (Murthy et al., 1982; Eakle et al., 1982; Arima and Bremel, 1983; Wallace, 1986). The MW of PLs in sheep (Martal and Djiane, 1975; Warren et al., 1990b) and goat (Currie et al., 1990) were found to be about 22,000, which was similar to the MW of hPL and monkey PL (Shome and Friesen, 1971). Clearly, the MW of bPL is different from PLs in all other known species, except for mouse PL, which exists in two forms of glycoprotein with different MW, ranging between 29,000-32,000 and 36,500-42,000 (Colosi et al., 1987).

Bovine PL has been shown to exist in at least three forms, which differ in isoelectric points and acidic AA compositions (Arima and Bremel, 1983). A structural comparison between bPL, oPL and cPL indicated that bPL contains unusual carbohydrate residues in its structure, including N-linked and O-linked oligosaccharides (Byatt et al.,

1990), whereas oPL and cPL are nonglycosylated polypeptides (Warren et al., 1990b; Currie et al., 1990; Anthony et al., 1995a). These observations suggested that glycosylation of bPL may account for the high MW and multiple isoelectric variants. Previously, it was suspected that glycosylation related to the multiple isoforms of bPL (Shimomura and Bremel, 1988). Subsequent studies observed that the number of isoforms does not change after deglycosylation (Byatt et al., 1990), thus variant genes are not responsible for these isoforms. Sequence analysis indicated that two splicing variants for bPL exist (Kessler and Schuller, 1991). This gave rise to the possibility that the existence of multiple isoforms of bPL may be involved in additional splicing patterns, thus translating different final products.

In cattle, recombinant (r) bPL has been produced in *Escherichia coli*, but unlike the native bPL, this is an unglycosylated protein hormone (Krivi et al., 1989). The effect of enzymatic deglycosylation of bPL on receptor binding and biological activity has been investigated using a somatotropin radioreceptor assay and the Nb2 lymphoma assay for lactogenic activity (Byatt et al., 1990). This data indicated removal of N-linked oligosaccharides from native bPL increased the binding of bPL to GH receptor (about 1.2 to 2.3 fold), whereas removal of O-linked sugar chains had no effects on the prolactin-like activity of bPL. Glycosylated and unglycosylated forms of bPL had slightly different affinities for the bGH receptor (R) and had no effect on lactogenic binding sites, suggesting that glycosylation may not be required for biological function of bPL. Thus the biological effects mediated by recombinant bPL may not be different from those mediated by native bPL hormone. Recombinant PLs in sheep (Colosi et al., 1989; Sakal

et al., 1997) and goats (Sakal et al., 1998) can also be prepared in large amounts, thus facilitating both *in vitro* and *in vivo* studies.

Biosynthesis of placental lactogen (PL)

In ruminants, the mode of delivery of PL into the maternal circulation is a process of migration and fusion of fetal binucleate cells (BCs) into the maternal endometrial epithelium. These cells are produced and developed in the fetal trophoderm. When they mature (fully granulated), they migrate out of the fetal tissue, across the fetal-maternal placenta interface, and fuse with maternal uterine epithelial cells. This leads to the formation of either transient trinucleate cells (TCs) in cattle, or persistent syncytium in sheep and goats (Wooding et al., 1981; Wooding, 1984; Wooding et al., 1986; Wooding et al., 1992; Duello et al., 1986; Morgan et al., 1990). Since the chorionic BCs in sheep produced an oPL messenger ribonucleic acid (mRNA) (Kappes et al., 1992), their migration appears to be essential to the transfer of the non-diffusible PL molecule across the placenta into maternal compartment. These findings also emphasize an important role of fetal BCs in the secretion and storage of PL.

There is little information concerning the mechanism by which PL is delivered into the fetal circulation. It has been established that the release of oPL into fetal circulation was probably controlled differently and was not derived from the maternal system (Schoknecht et al., 1991). Pregnant ewes and fetuses treated with ornithine, a potent stimulus of PL secretion, increased oPL concentrations in maternal plasma but not in the fetus (Grandis and Handwerger, 1983). The different effects of ornithine on both systems indicated discrete mechanisms for oPL secretion, into the two circulatory systems. The findings that radiolabeled oPL lacked the ability to cross the placental

barrier when infused into either maternal or fetal circulation (Reddy and Watkins, 1983), may support this interpretation. It is proposed that PL secretion into fetal circulation may result from either a population of BCs, which migrate across the placental barrier and release PL into both circulations, or from a population of fetal BCs that do not migrate across the placenta (Grandis and Handwerger, 1983; Kappes et al., 1992). Another possibility is that fetal PL is synthesized from some fetal organs and then directly secreted into fetal blood (Handwerger and Freemark, 2000).

Pattern of PL secretion

The pattern of PL secretion during pregnancy has been shown to be similar among species, including humans (Kaplan and Grumbach, 1965), sheep (Handwerger et al., 1977), goats (Currie et al., 1990), rats (Robertson and Friesen, 1981), mice (Soares et al., 1982) and cattle (Wallace, 1993). These PL hormones increase with advancing gestational age, peak during the last trimester, then decrease at or near parturition. However, the concentration of PL in maternal and fetal circulations is highly species dependent.

Maternal PL concentration: The expression of mRNA for bPL was observed in the bovine conceptus beginning at day 17 of pregnancy (Kessler et al., 1991), and was correlated with the appearance of fetal BCs (Flint et al., 1979). Maternal bPL secretion has been detected in the dairy cows as early as day 63 of gestation, with highest concentrations occurring at day 215, and remaining high until just before delivery (Wallace, 1993). The presence of oPL was detectable in the trophoblastic tissue at days 16-17 of pregnancy (Martal and Djiane, 1977). The concentration of oPL in maternal serum was first detected at day 41-50 (Handwerger et al., 1977), peaked at days 120-140

of gestation (Handwerger et al., 1977; Gluckman et al., 1979, Taylor et al., 1980), gradually declined thereafter, and was significantly lower on the day of delivery (Taylor et al., 1980). In goats, the first appearance of cPL was detected in maternal serum on day 44, peak concentrations occurred during the last third of pregnancy, and declined before parturition (Currie et al., 1990).

It has been suggested that, the pattern of bPL secretion in cattle is distinct from that of other species, even from closely related animals such as sheep and goats. The highest concentration of bPL (<3 ng/ml, Wallace, 1993) was much lower, as compared to peak maternal PL levels in other species, including in sheep (> 900ng/ml, Kappes et al., 1992), goats (1004ng/ml, Currie et al., 1990), mice (>250ng/ml, Soares et al., 1982), rats (400 ng/ml, Robertson and Friesen, 1981) and humans (> 4000 ng/ml, Sciarra et al., 1968). The reasons why levels of PL in the cow are much lower than those of other species are not fully characterized. Differences in migration patterns of the BCs among species, may explain the observed variations. It can be added that the rapid clearance rate of bPL from the blood, may also contribute for the low concentration of a hormone in the maternal circulation. The disappearance of recombinant bPL from maternal plasma was reported to be 7.25 min (Byatt et al., 1992c), which is thus removed from the circulation faster than most hormones reported, such as oPL (12.7 min, Reddy and Watkins, 1983), hPL (21-23 min, Beck et al., 1965), bGH (28min, Hart et al., 1980), bPRL (20-30 min, Akers et al., 1980). However, the serum-half life of native bPL may be longer because it is glycosylated (8.93 min, Torto, 1994).

Fetal PL concentration: The average concentration of bPL in fetal plasma was about 8 to 18 fold greater than that in maternal plasma (Holland et al., 1997). The highest

concentration of bPL in fetal blood ranged between 11.6-18.4 ng/ml, and gradually declined with advancing fetal age (Byatt et al., 1987). Although the concentration of bPL in fetal serum decreased throughout gestation, the level of bPL in the fetus is always higher than in maternal blood. Nevertheless, the much higher volume of maternal blood compared to that of the fetus, indicates large amounts of bPL are secreted into the maternal system.

Unlike in cattle, oPL in maternal blood exceeds the fetal level. Maternal serum concentration of oPL was 417.7 ng / ml on day 105, whereas fetal concentration was 29 ng/ml on day 90 of pregnancy (Kappes et al., 1992). Fetal oPL levels increased from days 60-120, peaked by mid-gestation, then stabilized or declined until term (Kappes et al., 1992; Gluckman et al., 1979; Taylor et al., 1980). Similar to sheep, the average concentrations of hPL in the fetus were approximately 350-fold less than those in maternal blood (Houghton et al., 1984).

Factors involving PL secretion: Possible factors involved in PL secretion in maternal and fetal serum include: stages of gestation, placental mass, fetal weight, litter size, the breed of fetuses and nutritional status. Concentrations of bPL in maternal serum increased as gestation progressed (Wallace, 1993). Secretion of bPL was affected by nutrient intake and body condition scores (BSC) with thin cows having greater plasma bPL concentrations than moderately conditioned cows, between days 200 and 256 of gestation (Rasby et al., 1990). Pregnant Holstein heifers fed a low crude protein (10.3 %) all forage diet had elevated bPL concentrations in maternal blood (Torto, 1994). Differences in maternal bPL concentrations were demonstrated in dams bearing different breeds of fetuses (Guilbault et al., 1990). Concentrations of bPL in maternal blood were

not affected by the number of fetuses being carried (Patel et al., 1996). Unlike cattle, serum PL concentrations in goats and sheep are highly correlated with litter sizes. Secretions of PL increased as the number of fetuses and placental weight increased in sheep (Butler et al., 1981) and goats (Hayden et al., 1980). Prolonged restriction of feed intake (72h) in pregnant ewes increased oPL concentrations both in maternal and fetal circulations (Brinsmead et al., 1981). Restriction of placental growth declined the production of oPL in the ewes and slightly declined fetal oPL levels (Falconer et al., 1985). Maternal plasma hPL increased significantly in pregnant women during fasting (Kim et al., 1971). Similar to sheep and goats, a significant correlation has been shown between fetal numbers, gestational stages and maternal hPL levels, greater PL concentrations were observed in multiple as opposed to singleton gestations (Spellacy et al., 1978). Serum hPL levels have also been correlated with placental weight, which is influenced both by the stage of gestation and number of fetuses, and the weight of the newborn at delivery (Sciarra et al., 1968; Houghton et al., 1984).

Mechanism of action of ruminant placental lactogens

It is well documented that PL is one of a group of polypeptide hormones, which are structurally related to pituitary GH and PRL. Similar to many protein hormones, PL initiates its biological action by binding to its specific receptor on the membrane of target cells. Since ruminant PLs have the ability to bind, and activate both PRL and GH receptors from other species, they probably have multiple biological effects similar to GH and PRL. However, the mechanism of action by which PL binds to its receptors, and the biological activities transduced as a result of this interaction, is not fully understood. It has been postulated that ruminant PLs may initiate their biological signals through

several possible ways: (1) activating a unique PL-R; (2) activating a variant form of GH-R; (3) dimerizing GH-R from heterologous animals, but not from homologous (ruminant) species; (4) heterodimerizing GH and PRL receptors in ruminants; (5) transducing the signals through PRL receptors in both heterologous and homologous species.

Activating a unique PL-R: Ruminant PLs may initiate biological activity through a specific receptor, which is structurally distinct from the GH-R. Specific binding sites for bPL, but not for bGH, were identified in the uterine endometrium of pregnant cows, suggesting bPL may act through a distinct bPL-R in the endometrium, to maintain pregnancy (Galosy et al., 1991). Specific receptors for bPL were also found in membranes isolated from bovine luteal tissue that displayed high affinity for bPL, but very low affinity for either bGH or bPRL (Lucy et al., 1994). In contrast, both bGH and bPL bound to bGH-R in the hepatic bovine membrane with high affinity (Byatt et al., 1995). These findings raise a possibility that in liver tissue, bPL may exert its actions through either bGH or bPRL receptors, whereas in the uterus and corpus luteum (CL) membranes, bPL may act through a unique PL –R.

An *in vivo* study with mature female rats has shown that bPL exhibited an acute effect on weight gain at a low dose, and increased feed intake to a greater extent, as compared to bGH (Byatt et al., 1991). Studies in dairy cows reported bPL displays distinct effects on intermediary metabolism (Byatt et al., 1992c) and feed intake (Byatt et al., 1992b), as compared to bGH. These observations indicated that bPL might work through a mechanism that is different from that of bGH.

In sheep, the presence of a specific oPL-R in the liver of fetuses and pregnant ewes was also reported (Freemark and Handwerger, 1986; Freemark et al., 1987). In

ovine fetal tissues, oPL-stimulated glycogen synthesis occurred to a greater extent (18-167%), as compared to oGH (12%) and oPRL (4%) (Freemark and Handwerger, 1986). Specific binding for oPL was further shown in CL in sheep (Chan et al., 1978). A distinct effect of oPL on food intake, as compared to bGH, was also observed in young lambs (Min et al., 1996). Subsequent reports demonstrated oPL has an indirect effect on the fetal somatotrophic axis, by increasing the expression of insulin-like growth factor binding protein 3 (IGFBP-3) in the fetal liver, whereas oGH treatment does not have comparable effects (Currie et al., 1996). These data proposed that the metabolic effects of oPL might be mediated through binding to a specific PL-R.

Activating a variant form of GH-R: Another possibility is that ruminant PLs may act by homodimerizing of a modified GH-R, which is mutated at its extracellular domain (ECD) in a way that allows dimerization of GH-R. Two types of mRNA (4.7 and 4.4 kb) for GH-R were found in bovine CL and ovary, whereas only a single type (4.4 kb) was detected from the liver (Lucy et al., 1993). Further investigations suggested that bPL may bind to the CL through a modified (4.7 kb) form of the bGH-R (Lucy et al., 1994). In addition, in the bovine liver, the ECD of bGH-R binds to bGH and bPL with high affinity, but with different stoichiometry (Staten et al., 1993). While bGH binds to the bGH-R in a 1:2 ratio (1bGH: 2bGH-R), bPL does not cause homodimerization of the bGH-R. These data suggested that the ECD of bGH-R could be modified, and these conformational changes, increase binding specificity for bPL, but significantly reduce affinity for bGH (Byatt et al., 1995).

Overall, available evidence indicates the presence of specific ruminant PLs, and some efforts have been made to purify and characterize the distinct PL-R (Freemark and

Comer, 1989). However, the unique PL-R has not yet been isolated up to date. Thus, the question of whether the unique PL-R exists or whether ruminant PLs work through a modified GH-R remains unsolved.

Homodimerizing of GH-R from homologous species: Research has shown that in heterologous species (rats, rabbits, humans), ruminant PLs can mimic GH actions by dimerizing GH-R. However, in homologous (ruminant) systems, PLs can bind to, but not homodimerize ruminant GH-Rs. Utilizing *in vitro* heterologous systems, using rat 3T3-L1 adipocytes to examine the somatogenic activity, Byatt and coworkers (1991) found both bPL and bGH are similar in their ability to inhibit the insulin-stimulated glucose uptake. A further study reported that in 3T3-F442A rat preadipocyte bioassays that the somatogenic activity of bPL is slightly less potent (six-fold lower) than hGH (Vashdi et al., 1992). However, in primary cultures of rat hepatocytes, bPL was approximately 100-fold more potent than bGH in inducing an insulin-like growth factor I (IGF-I) mRNA expression (Warren et al., 1993). IGF-I and IGF-II are characterized as serum polypeptides, which play an important role in regulating growth. Similar results have been observed in sheep and goats. The mitogenic activity of oPL or cPL was as potent as hGH in two heterologous bioassays using FDC-1 cells transfected with rabbit or human GH receptors (Sakal et al., 1997; Sakal et al., 1998). Interaction studies have demonstrated that, bPL is capable of forming 1:2 complexes with hGH-R (Vashdi et al., 1995; Helman et al., 1998) and rabbit GH-R (Helman et al., 1997). Gel-filtration experiments also observed oPL and cPL form 1:2 complexes with rabbit GH-R or hGH-R (Sakal et al., 1997; Sakal et al., 1998, Helman et al., 1998). Results of these data clearly

documented that ruminant PLs have capacity to homodimerize GH-Rs in heterologous species.

Results from studies with rats have proven that bPL displays more potent somatogenic activity (stimulating weight gain) as compared to bGH (Byatt et al., 1991). Similar to cattle, oPL also showed a greater growth-promoting effect, as compared to bGH, in GH-deficient dwarf rats (Singh et al., 1992). In contrast, studies with cattle have shown bPL could not mimic all of the metabolic effects of bGH, indicating that in homologous species bPL only acts as a weak GH agonist (Byatt et al., 1992b). The fact that in the bovine liver bPL interacted with bGH-R with about three-fold less potency than bGH (Byatt et al., 1991), may be one of the relevant reasons to explain why bPL only displays partial somatogenic responses in dairy cows as compared to bGH.

There has been controversy in reporting that bPL has somatogenic activity *in vivo*, but does not induce homodimerization of the bGH-R. A number of studies have been performed to reexamine the activity of ruminant PLs through homologous and heterologous GH-Rs. A homologous bioassay was carried out using two cell lines Ba/F3-Cl and BHK-24, which are transfected with bGH-R, to evaluate proliferative and cellular signaling differences between bPL and bGH (Warren et al., 1999). The results showed that bGH stimulates mitogenic activity and phosphorylation of JAK2, a tyrosine kinase responsible for the signal transduction downstream of the receptor, while bPL was not involved in either process, despite both hormones binding to bGH-R with a similar affinity. These findings were consistent with previous experiments using a murine lymphoid cell line, Baf/3 (Warren et al., 1997). The lack of biological responses resulting from bPL binding to the bGH-R indicated that this might be due to an inability of bPL to

induce dimerization of bGH-Rs. These observations suggested that homodimerization of bGH-R may be essential for these biological activities (Warren et al., 1999).

Cunningham and coworkers (1991) used the human GH model system to demonstrate that the sequential dimerization is important for activation of cytokine receptors. They observed that the hGH molecule has two separate sites for receptor binding, termed site 1 and site 2. In order to produce an active homodimer (1 hGH : 2 hGH-R), at first GH-R binds to site 1 on GH, and subsequently at site 2 to on the same molecule of GH. This active molecule was required for intracellular signaling by the GH-R and for phosphorylation of cellular proteins through activation of tyrosine kinase (Silva et al., 1993). However, excess hGH antagonized signaling by preventing homodimerization. This leads to a formation of an inactive 1:1 complex, which acts as a somatotropin antagonist. Similarly, Fuh and his coworkers (1992) also observed mutant GH, which lacks binding site 2 and possesses only site 1, can antagonize signaling by preventing dimerization. They also found the proliferation of FDC-P1 cells is lost at very high hGH concentrations. Later studies by Herman and coworkers (1999) supported previous findings. They demonstrated that oPL is inactive or antagonistic to the mitogenic activity of oGH in homologous bioassays using “293” cells transfected with full size oGH-R. The activity of cPL and bPL was similar to that of oPL. In contrast, all three PLs acted as agonists in heterologous bioassays in cells transfected with either rabbit GH-R or human GH-R. Although these hormones differ in their activities, oGH and PLs bind to oGH-R with similar affinity, indicating that the binding occurs through site 1 of the hormones. This interpretation is consistent with most studies, implying that ruminant PLs form only 1:1 complexes with oGH-R (Herman et al., 1999), or bGH-R

(Staten et al., 1993). Taken together, these data support a new theory, that in homologous systems, ruminant PLs antagonize the mitogenic activity of GH because they do not homodimerize GH-Rs, whereas in heterologous species they do, and thus act as an agonist.

Heterodimerizing of GH and PRL receptors from ruminant animals: Another assumption has implicated that ruminant PLs may transduce biological signals by heterodimerization of homologous GH and PRL receptors. An experiment was conducted to investigate whether ruminant PLs can initiate a biological signal by heterodimerizing GH and PRL receptors (Herman et al., 2000). A significant result of this experiment was that a triple complex with a high molecular mass consisting of three components of oPL, oGH-R and bPRL-R was detected (bPRL-R was used instead of oPRL-R since these proteins have a high percentage of similarity (96%) and exhibit similar activity in cells transfected with PRL-R). These results were obtained by using a novel method, based on surface plasmon resonance (SPR), to measure the real-time kinetic interaction between molecules and the stoichiometry of a complex (Johnsson et al., 1991). It has been described that the heterotrimeric complex is formed by consecutive binding of oGH-R through site 1 of immobilized oPL and bPRL-R through site 2 of the same oPL molecule. Their results also showed that oPL or bPL significantly enhances bioactivity in cotransfected cells with oGH-R and oPRL-R, while little or no biological signal is observed in cells transfected with oGH-R or oPRL-R alone. Subsequent SPR studies also indicated the kinetics of interactions are different (Gertler et al., 2002). Their results indicated that whereas the 1:1 (oPL:oGH-R) complex has a half-life of about 50 min, the interaction between the 1:1 complex and bPRL-R is very unstable, with a half-life of less

than 0.2 min. This transient complex has been detected in SPR studies, but not by gel-filtration experiments, due to its rapid dissociation to the 1:1 form (Gertler et al., 1996). Recent work by Biener et al., (2003) confirmed a unique property of oPL in its ability to heterodimerize homologous GH-R and PRL-R. Together, these data provide evidence that heterodimerization between two distinct cytokine receptors does occur and leads to initiation of signal transduction.

Additional *in vivo* evidence, supporting the new hypothesis reported that co-administration of bGH with oPL to postnatal lambs for 5 days elevated IGF-I levels more than that stimulated by bGH or oPL alone (Ogawa et al., 1995). Short-term (9 d) administration of bPL to dairy cows increased milk yield, although bPL was less potent than bGH (Byatt et al., 1992b). Lactating ewes treated with oGH or oPL for 14 days increased milk yield, but oGH was significantly more potent than oPL (Leibovich et al., 2001a). Since ruminant PLs only mimic partial GH-like galactopoiesis, it is thought that these effects are likely achieved by the heterodimerization of PL with homologous GH and PRL receptors.

Activating PRL-Rs from both heterologous and homologous species: It has been suggested that ruminant PLs may act by activating PRL-Rs in both homologous and heterologous systems. Accumulating evidence indicates that ruminant PLs have an ability to act in cells possessing both homologous and heterologous PRL receptors. In an vitro heterologous bioassay, bPL had the capacity of acting through PRL-Rs to stimulate proliferation of rat Nb2 lymphoma cells (Byatt et al., 1990). Bovine PL also stimulated α -casein (milk protein) synthesis in mammary gland explants from pseudo-pregnant rabbits (Bignon et al., 1994). Lactogenic activity of oPL and hGH were equipotent in Nb2 cell

proliferation bioassay (Sakal et al., 1997). The synthesis of β -casein by oPL and cPL was significantly greater than that of oPRL in rabbit mammary gland explants (Sakal et al., 1997; Sakal et al., 1998). By contrast, in homologous bioassays, in ovine mammary gland explants the β -casein production induced by oPL was slightly less potent, as compared with oPRL (Sakal et al., 1997). A recent homologous bioassay has also confirmed that, the lactogenic activity of oPL, is less potent than that of oPRL in HEK 293 cells transfected with oPRL-R (Leibovich et al., 2001b).

To gain further knowledge, some researchers have investigated the interaction of various lactogenic hormones with PRL-Rs prepared from several species, using both gel filtration and SPR experiments. Their results showed that bPL forms 1:2 complexes with PRL-R in both heterologous and homologous species, such as rabbits (Bignon et al., 1994), rats (Tchelet et al., 1995; Helman et al., 1998) and the cow (Gertler et al., 1996). Similar results were observed with oPL (Sakal et al., 1997; Helman et al., 2001) and cPL (Sakal et al., 1998). The fact that ruminant PLs are able to interact with various PRL-Rs in both homologous and heterologous systems to form a homodimer, together with the previous experiments, which noted that ruminant PLs are lactogenic *in vivo*, suggests that ruminant PLs can act through homologous PRL-Rs, although they are not a potent lactogen in homologous species. These results could be due to the lower binding affinity of oPL to ewe mammary glands, as compared to rabbit mammary tissues (Servely et al., 1983), and due to lower affinity of oPRL, bPRL, bPL to bPRL-R, as compared to hGH (Tchelet et al., 1995; Sakal et al., 1997). Some recent studies have demonstrated the mechanism of ruminant PL actions that gave rise to another possible explanation that in homologous species ruminant PLs may act by heterodimerizing GH and PRL receptors,

thus exhibiting bioactivities that are distinct from that of PRL or GH. Whereas in heterologous species, the PL signal may be transduced through homodimerization of GH-R, thereby displaying similar GH-like galactopoiesis.

To better understand the differences between the activities transduced through heterologous and homologous PRL-Rs, a novel hypothesis has been developed, namely “a minimal time of homodimer persistence” (Helman et al., 2001). It is assumed that, in order to initiate the hormonal signal transduction, the associated kinase JAK2 has to be transphosphorylated and this requires a minimal time of homodimer existence. In the case of homologous interactions, even though the transient dimerization of PRL-R with ruminant PLs lasted only a few seconds, this minimal time was still sufficient to elicit a full biological response (Gertler et al., 1996). Thus, ruminant PLs are able to act via the lactogenic receptor, in both homologous and heterologous systems, despite the fact that the binding properties and stoichiometries of interaction in homologous species are different from those of heterologous systems.

Biological functions of ruminant placental lactogens

The biological functions of PLs are generally not fully understood. They probably have multiple biological effects related to luteotrophic activity, mammogenesis, lactogenesis, somatogenesis and fetal development.

Luteotrophic action: Factors that stimulate corpus luteum (CL) function are referred to as luteotrophic. Progesterone secreted by the CL is essential for the maintenance of early pregnancy in all farm species. In sheep, after day 50 the ovary is no longer required for the maintenance of pregnancy, since the placenta secreted large amount of progesterone

(Bassett et al., 1969). By contrast, in goats, cows and rodents the CL alone is responsible for progesterone throughout the whole pregnancy.

It has been reported that ovarian function can be modified by bGH, and similar changes may occur with bPL in the cow. During an estrous cycle, administration of bGH increased the number of ovarian follicles in lactating cows (Delasota et al., 1993) and heifers (Gong et al., 1991). Injections of recombinant bGH to lactating dairy cows increased plasma progesterone concentrations (Schemm et al., 1990; Gallo et al., 1991), and the weight of the CL (Lucy et al., 1992). The presence of bGH-R was detected in both bovine CL (Scott et al., 1992) and ovary (Lucy et al., 1993). These findings inferred that bGH might have direct effects on the bovine ovary and CL through binding to GH-R and these effects may correlate with an increase in circulating IGF-I concentrations (Peel et al., 1987).

Later work found that both recombinant bGH and bPL have stimulatory effects on the development of the CL and ovarian follicles in heifers, but their effects appear not to be identical (Lucy et al., 1994). While bPL displayed higher potency in stimulating CL growth and progesterone secretion, the potency of bGH was greater in stimulating the IGF-I concentration and the second largest follicle development. Both hormones caused a similar effect in inhibiting preovulatory follicle development. The existence of specific binding sites, which displayed high-affinity for bPL, but not bGH (Lucy et al., 1994), together with the presence of a modified mRNA for bGH-R found within the CL (Lucy et al., 1993), caused speculation that, bPL may act by binding to an altered form of the bGH-R to support CL development and inhibit follicular growth during pregnancy. This hypothesis, however, is still being debated.

Reports on the luteotrophic role for oPL have been conflicting. Early work with ewes had shown the presence of a specific binding site for oPL in ovine CL membranes (Chan et al., 1978). This report was in contrast to other experiments, which showed oPL does not have the ability to stimulate progesterone secretion, or to protect the CL against the luteolytic action of prostaglandin F ($\text{PGF}_{2\alpha}$) when infusing oPL for 2h into the ovary of the sheep (Schramm et al., 1984). A later experiment indicated immunoneutralization of oPL for 12h has no effect on progesterone concentration in late pregnant ewes (Waters et al., 1985). A recent experiment confirmed the luteotrophic and luteoprotective actions of oPL on the CL obtained from pregnant ewes (Gregoraszczuk et al., 2000). Their results showed that oPL stimulated luteal cells isolated on days 45 and 70 of gestation to produce progesterone, suggesting a direct luteotrophic effect on the CL during the early half of pregnancy. This data was consistent with the results of Chan and his co-workers (1978), who suggested oPL binds specifically to the ovine CL membrane. Other reports also emphasized that oPL is luteotrophic on luteal cells, obtained at days 40-45 of gestation whereas both oGH and oPRL do not cause significant effects (Wierzchos et al., 2000). Additionally, it has been observed that co-administration of oPL and $\text{PGF}_{2\alpha}$ significantly increased progesterone secretion by luteal cells isolated on days 70 and 95 of pregnancy whereas a $\text{PGF}_{2\alpha}$ treatment alone decreases progesterone secretion, which implies the role of oPL against luteolytic action of prostaglandin (Gregoraszczuk et al., 2000). Further evidence has been given in rodents, indicating the mouse ovary contains receptors that specifically bind to mouse PLs (MacLeod et al., 1989) and maintain progesterone secretion during the later half of pregnancy (Galosy and Talamantes, 1995; Thordason et al., 1997). Thus, available information reported from various species

indicates the important role of PLs on the CL function during pregnancy. However, the mechanism of action controlling this process remains unclear.

A homologous bioassay, using HEK 293 cells transfected with either oPRL-R or primarily luteal cells, was conducted to compare the luteotrophic activity of oPL with oGH and oPRL (Leibovich et al., 2001b). These results showed that oPL significantly increased progesterone secretion in ovine luteal cells collected on days 17 and 45 of pregnancy, whereas oPRL and oGH had no such effects. On the other hand, all three hormones further increased progesterone secretion (about seven to eight fold), in the primary culture of luteal cells obtained from mid-pregnant ewes. Since both oPRL and oGH were inactive in stimulation of progesterone production during early pregnancy, these observations suggested that in early gestation, the luteotrophic effects of oPL are probably obtained via heterodimerizing PRL and GH receptors. Later in gestation (midpregnancy), however, the luteotrophic effects of oGH and oPRL were achieved through activating their unique receptors, whereas oPL acted, either by activation of PRL-Rs, or heterodimerizing homologous GH and PRL receptors (Leibovich et al., 2001b).

Mammogenesis: Mammary growth (mammogenesis) is known to occur predominantly during late pregnancy, when PL is at a high concentration, indicating this hormone may be related to mammogenesis and lactogenesis. It is well known that the lobulo- alveolar cells of the mammary gland are stimulated by PRL, thus depression of PRL concentrations by bromocryptine treatment inhibited mammary development in non-pregnant-ewes (Schams et al., 1984). During pregnancy, however, bromocryptine administration did not affect mammary development of sheep, cattle (Schams et al., 1984)

and goats (Forsyth et al., 1985). These data suggest that ruminant PLs may act on the mammary glands and may substitute for prolactin to support mammary growth during pregnancy.

In cattle, bPL increased deoxyribonucleic acid (DNA) synthesis in bovine mammary transplants maintained in nude mice, suggesting a mammogenic role for bPL (Vega et al., 1989). In addition, rbPL has been shown to stimulate total mammary DNA in dairy heifers, but this effect does not occur in groups treated with a high level of rbPRL (80 mg/d) (Byatt et al., 1994). They also observed that both hormones induce mammary differentiation (milk synthesis), although bPRL appears more potent than bPL. Further evidence indicated that PRL is required for lactogenesis rather than for mammary growth in periparturient cows (Akers et al., 1981; Collier et al., 1993). These results suggested that bPL stimulates mammogenesis and that a high level of prolactin is not required for mammary growth in cattle. Moreover, a positive relationship between rbPL concentration and milk production was found in dairy heifers (Byatt et al., 1997). These data support the hypothesis that bPL is one of the factors that is involved in mammary development during pregnancy.

Although bPL has been shown to bind to bPRL-R with high affinity (Scott et al., 1992), this hormone also binds to bGH-R (Byatt et al., 1990). It is not clear whether the mammary response to bPL is mediated directly by the mammary tissue or via an indirect mechanism. Collier and coworkers (1993) demonstrated that PL, GH and PRL do not appear to affect mitogenic activity of bovine mammary epithelial cells (BMEC) *in vitro*, using mammary tissue obtained from non-lactating heifers at 150 days of pregnancy. However, GH and PL increased BMEC DNA synthesis in late pregnant cows or dairy

heifers when directly infused into the mammary gland. On the other hand, IGF-I displayed a positive effect both *in vitro* and *in vivo*. These results suggested the effects of GH and PL on mammary growth are probably regulated via members of the somatomedins, rather than directly via a lactogenic receptor pathway. These explanations are supported by the direct *in vitro* study of IGF-I that demonstrated that IGF-I and-II increased proliferation of BMEC (McGrath et al., 1991). In dairy cows, rbPL also increased IGF-I and II concentrations, despite being less potent than bGH (Byatt et al., 1992c). Furthermore, rbPL was more active than rbPRL in stimulating mammary secretion of IGFBP-2 and -3 (Byatt et al., 1994). Clearly, members of the somatomedins are important mediators of mammary epithelial growth in ruminants.

In sheep, oPL was very efficient in stimulating β -casein synthesis from the mammary glands of rabbits but not from ewes (Severly et al., 1983; Sakal et al., 1997). Similar results were reported for PL in goats (Sakal et al., 1998). In contrast to cattle, oPL was unable to compete for prolactin binding sites in the ovine mammary gland (Emane et al., 1986). These observations suggested that oPL might not play an important role in mammogenesis and lactogenesis in ewes. Later work has emphasized that recombinant oPL and oGH have mammogenic effects in ewes, via stimulating the development of lobulo-alveolar systems (Kann et al., 1999). They also found, both hormones increase plasma IGF-I concentrations, although oPL is less efficient than oGH. These results agreed with other experiments indicating that IGF-I increased DNA synthesis of ovine mammary cell in cultures (Winder et al., 1989). Together, these data suggest that oPL and oGH are mammogenic in ewes and IGF-I could be one of the mediators of these hormones.

Lactogenesis: Factors that stimulate the mammary gland to synthesize milk proteins are defined as being lactogenic (Soares et al., 1991). *In vitro* studies across species have demonstrated that bPL specifically binds to PRL-R in rabbit liver membranes (Eakle et al., 1982) and rabbit mammary explants (Byatt and Bremel, 1986), suggesting bPL is lactogenic in this species. The potency of bPL was comparable to that of bPRL in the rabbit Nb2 cell proliferation bioassay (Murthy et al., 1982). In IM-9 human lymphocytes, bPL was as potent as hGH in its ability to bind to hGH-R (Vashdi et al., 1995). By contrast, in homologous bioassay, using mammary gland explants from pregnant heifers, bPL failed to stimulate the production of lipid, casein and α -lactalbumin, which is required for lactose synthesis, suggesting bPL is not galactopoietic in the cow (Byatt and Bremel, 1986). Galactopoiesis is defined as milk synthesis during established lactation.

Studies in dairy cows have proven that short-term (9d) administration of bPL and bGH increase milk yield (Byatt et al., 1992b). A later study reported injections (7 days) of bPL or bPRL into dairy heifers stimulated mammary differentiation, such as increasing the serum concentration of α -lactalbumin (Byatt et al., 1994). Bovine PL also stimulated milk yield of non-pregnant lactating cows and heifers after 7 to 9 days of treatment (Collier et al., 1995). In all cases, bPL appears to be less active than bGH and bPRL. These data strongly suggest that bPL is lactogenic, however the role of bPL in stimulating milk production may not be critical for the cow.

The lactogenic activity of oPL has been investigated in mammary glands from rabbits and ewes (Servely et al., 1983). These data reported that oPL is more potent than oPRL in accumulating β -casein mRNA in mammary glands from rabbits but not from ewes. These findings provide further evidence that this placental hormone is not a potent

lactogen in the homologous species. Since oPL binds with lower affinity to lactogenic receptors from ovine mammary gland explants, as compared to the rabbit mammary tissue (Sakal et al., 1997), this may explain why oPL is a less potent stimulator of β -casein and fatty acid synthesis, as compared to oPRL.

There is conflicting evidence on the lactogenic activity of oPL in ewes. Min and his coworkers (1997) observed administration of bGH to the ewe for 5 days increased milk yield and milk composition. Serum concentrations of IGF-I and non-esterified fatty acid (NEFA) were elevated, but such effects were not observed in oPL-treated ewes, suggesting oPL is not lactogenic in sheep. Subsequent studies reported that treatment of bGH or oPL to lactating ewes for 5 days did not affect GH-R, IGF-I or IGFBP-3 mRNA in the mammary tissue or the number of GH-R in the liver. In addition, milk yield and hepatic expression of IGF-I mRNA increased following bGH but not oPL treatment (Bassett et al., 1998). These observations suggested that the effect of bGH on milk yield is partially mediated by an endocrine action of IGF-I, and oPL is not lactogenic in lactating ewes. It is speculated that the absence of plasma IGF-I concentrations during a short-term (5d) treatment period in ewes may be responsible for the lack of oPL effect on milk production. The findings that IGF-I significantly increased milk secretion, when infused directly to the mammary artery of goats (Prosser et al., 1990), together with the study reporting that bPL increased both milk yield and IGF-I in dairy cows treated with bPL for 9 days (Byatt et al., 1992b), may support this speculation.

A thorough examination of the lactogenic role of oPL in pregnant ewes was performed due to the previous conflicting evidence (Kann et al., 1999). Their results indicated that ewes treated with oPL for 10 days increased circulating IGF-I, and β -casein

mRNA accumulation in ovine mammary explants. In goats, recombinant cPL also induced β -casein synthesis in rabbit and ovine mammary glands. However, cPL action was more potent than oPRL in mammary gland cultured from rabbits, but not from ewes (Sakal et al., 1998). Later work reported administration of oGH or oPL into mid-lactation ewes for 14 days increases milk production by up to 50% and 25%, respectively. Serum IGF-I concentrations were elevated by bGH, but not by oPL treatment (Leibovich et al., 2001a). These findings agreed with most studies, reporting that oPL treatments do not affect the circulating IGF-I concentrations in sheep (Ogawa et al., 1995; Min et al., 1997; Bassett et al., 1998). These data suggested that both oGH and oPL display lactogenic effects in lactating ewes, but these hormones do not have the same mechanism of stimulating milk production, because only oGH treatment resulted in an increase in serum IGF-I concentrations.

As stated previously, oPL displayed low activity in cells transfected with oPRL-Rs and no activity in cells transfected with oGH-Rs, but significantly greater activity in cells transfected simultaneously with both types of receptors (Herman et al., 2000). Together with the results that have been mentioned above, it could be interpreted that lactogenic effects of oPL (also likely for other ruminant PLs) are achieved either through homologous PRL-Rs, or through heterodimerization of homologous GH and PRL receptors (Leibovich et al., 2001b), and that IGF-I could be one of the mediators involved in this process (Kann et al., 1999).

Somatotropic action: It is known that all three ruminant PLs have a low identity with GH, but they are able to interact with the GH (somatogenic) receptor from various species, thus displaying somatogenic activities both *in vitro* and *in vivo*. Studies across

species have discovered that bPL binds to the GH-R in the liver membranes from rabbits (Eakle et al., 1982), cattle (Wallace and Collier, 1984; Staten et al., 1993), sheep (Emane et al., 1986), and ovine fetuses (Freemark and Handwerger, 1986). The binding affinity of oPL to the oGH-R in fetal and adult sheep liver was observed to be similar or greater than for oGH (Breier et al., 1994). Both cPL and oPL formed 1:2 complexes with hGH-R, and rabbit GH-R (Sakal et al., 1998). These data provide evidence that ruminant PL have the ability to bind to GH-R from a number of species, and thus mimic some somatogenic effects similar to GH.

Some investigators have proposed that ruminant PLs have potent somatogenic actions in heterologous species, but only display partial activities in homologous systems. In somatogenic bioassays, using either 3T3-L1 or 3T3-F442A rat-derived preadipocyte cells, the antimitogenic effect (Vashdi et al., 1992) or inhibition of glucose uptake (Byatt et al., 1991) by bPL was slightly less potent or similar to that of hGH. In a rat hepatocyte culture, bPL was 100-fold more potent than bGH in inducing IGF-I mRNA expression (Warren et al., 1993). In mature female rats, rbPL was more active than bGH in stimulating weight gain and feed intake (Byatt et al., 1991). These effects were obtained without a concomitant increase in plasma IGF-I levels, as occurred with bGH. Based on the observations that bPL had a capacity of binding to both GH (Staten et al., 1993) and PRL (Scott et al., 1992) receptors, together with the report indicating that PRL significantly stimulated feed intake in rats (Gerado-Getters et al., 1989), it appears that bPL stimulates weight gain through a somatogenic mechanism or through another route, probably by lactogenic receptors. In sheep, the somatogenic activity of oPL was described in GH-deficient dwarf rats, where oPL and oGH are equipotent in stimulating

serum IGF-I concentrations (Singh et al., 1992). Together, these findings suggest that ruminant PLs may bind and function through the GH-R in heterologous (rat) species.

Contrasting results have been obtained regarding the different somatogenic effects of ruminant PLs between heterologous and homologous species. Studies in dairy cows have found that bPL does not mimic all somatogenic effects of bGH (Byatt et al., 1992b; Byatt et al., 1992c). Their results showed that administration of bGH to pregnant and non-pregnant dairy cows increased NEFA, glucose and insulin, whereas bPL failed to display any effects on these parameters. Both bGH and bPL increased IGF-I, but bPL was much less potent than bGH. In addition, both hormones decreased blood urea nitrogen, indicating increased nitrogen retention. The evidence indicated that, bPL only displays several somatotrophic effects as compared to bGH (Byatt et al., 1992a, 1992b), and bPL binds to GH-R in the bovine liver about 1.8 to 3.2 fold less potently than bGH (Byatt et al., 1991). This may explain why bPL acts as a partial somatotropin agonist in homologous systems.

Another experiment with 2-month-old lambs has shown that, administration (5 days) of bGH or bGH + oPL, but not of oPL alone, significantly increased circulating IGF-I concentrations, suggesting that oPL alone lacks somatogenic actions in postnatal lambs (Ogawa et al., 1995). In contrast, administration of roPL for 21 days increased growth and energy intake in 3-day-old lambs (Min et al., 1996). Other reports indicated the growth-promoting effects of roPL were similar to oGH when injected to 2-month-old lambs for 35 days (Leibovich et al., 2001a). The differences in these reports may be explained by differences in treatment periods, the age of lambs and experimental designs.

As discussed before, homodimerization of the GH-R must occur, in order for the ligand-receptor complex to signal a biological response (Warren et al., 1999). This indicates that PL can simultaneously bind to both ruminant GH-R and PRL-R (Herman et al., 2000; Leibovich et al., 2001b). The co-administration of bGH and oPL to postnatal lambs significantly increased serum IGF-I concentrations, whereas bGH or oPL treatment alone had no such effects (Ogawa et al., 1995). Additionally, oPL enhanced the plasma levels of IGF-I in ewes even after 2 days of treatments (Kann et al., 1999). It is emphasized again that in heterologous species, ruminant PLs display somatogenic activities, which are most likely mediated through GH-R. In homologous systems, however, ruminant PLs act by heterodimerizing of GH and PRL receptors. IGF-I has been suggested as one of the mediators regulating of these effects.

Fetal growth – maternal and fetal metabolism: Placental lactogen has been shown to be present in high concentration in the fetus during the second half of gestation (Hill et al., 1988; Byatt et al., 1987; Kappes et al., 1992), suggesting PL may play a role in the regulation of fetal growth. Most of the information, regarding fetal and maternal metabolism is from experiments performed in sheep and humans. It is proposed that the effects of PL on fetal metabolism are mediated directly via fetal tissues, and indirectly via changes in maternal metabolism (Handwerger, 1991). Results from *in vitro* studies have indicated that oPL enhances the activity of ornithine decarboxylase (ODC) in the liver of rat fetuses, whereas this variable is not affected by oGH, oPRL, rat GH and rat PRL (Hurley et al., 1980). These results suggested an important role for oPL in the regulation of fetal growth via ODC, a vital enzyme for controlling cellular growth. In addition, oPL stimulated IGF-II synthesis in fetal rat fibroblasts (Adams et al., 1983), amino acid (AA)

transport in fetal rat diaphragm (Freemark et al., 1982 & 1983) and glycogen storage in the fetal rat liver (Freemark and Handwerger 1984 & 1985), while oGH displayed little or no effects on these parameters. In the ovine fetal liver, oPL promoted glucose incorporation into glycogen with a greater potency than was seen with oGH and oPRL (Freemark and Handwerger, 1986). The anabolic effects of human PL on fetal metabolism have been implicated, for example, promoting AA uptake, DNA synthesis and IGF-I production in human fetal myoblasts, fibroblasts and hepatocytes in culture (Hill et al., 1986; Strain et al., 1987). The influence of rat PL on embryonic growth and development was recently noted (Seoyum et al., 1999). The observations that GH has no significant effect on fetal growth and metabolism, when infused in late-gestational fetal sheep for 10 days (Bauer et al., 2000), together with the lack of GH-R in fetal tissues until term (Freemark and Handwerger, 1986), suggest that PL may function as a “fetal growth hormone”.

Concentrations of hPL in the fetus have shown to correlate positively with fetal IGF-I, IGF-II and IGFBP levels, suggesting the role of PL in fetal IGF synthesis (Lassarre et al., 1991). Another report was consistent with previous findings, implying infusion of oPL into the ovine fetus for 14 days, at a late stage of gestation (day 122), elevated serum IGF-I concentrations and hepatic glycogen deposition (Schoknecht et al., 1996). A study with pregnant ewes has demonstrated that, roPL treatment does not increase IGF-I in either the mother or fetus, but significantly increases the IGFBP-3 gene expression in the fetal liver, suggesting oPL may have an indirect effect on fetal growth by changing IGFBP-3 production (Currie et al., 1996). Both IGF-I and IGF-II have been

demonstrated as important fetal growth factors (Owen et al., 1991), thus PL in the fetus may be a regulator of fetal growth by controlling IGF-I and IGF-II secretion.

Several researches have pointed out that, PL may regulate the partitioning of maternal nutrients to support fetal growth, perhaps via IGFs, in humans (Handwerger et al., 1991) and sheep (Anthony et al., 1995a). Contradictory results have been reported on maternal and fetal metabolism in sheep. Intravenous infusion (8h) of partially purified oPL into fasting pregnant and non-pregnant ewes reduced NEFA, glucose and amino nitrogen levels, but elevated plasma insulin concentrations (Handwerger et al., 1976). Conversely, infusion (36h) of an ovine placental extract, enriched in oPL, to non-pregnant ewes induced opposite effects, with no effects on insulin (Thordarson et al., 1987). Another study reported that antibody neutralization of maternal oPL (12h) significantly increased plasma insulin levels, reduced free fatty acid (FFA) concentrations, and had no effect on body glucose metabolism (Waters et al., 1985). The conflicting results could be interpreted as differences in nutritional status of the ewes, treatment periods and experimental models. Nevertheless, these results show that oPL may modify maternal metabolism to provide nutrients for fetal growth.

Additional data have demonstrated the role of PL in the metabolic adaptation during pregnancy. Prolonged fasting (72h) of pregnant ewes increased oPL concentrations in both maternal and fetal serum (Brinsmead et al., 1981), and reduced the number of PL receptors in the maternal and fetal liver (Freemark et al., 1989 and 1992). However, by infusing glucose into fasted ewes, only the numbers of receptors in the fetal liver were reversed (Freemark et al., 1992). This may indicate the metabolic adaptation of the mother during nutritional stress, thus providing sufficient nutrients that are used to

support the developing fetus. Torto (1994) found Holstein cows fed with low protein, all-forage diets also increased maternal bPL concentrations. Pregnant women, who fasted for 84-90 hours during mid-pregnancy, increased their plasma hPL concentrations, coincident with a rise in FFAs (Kim et al., 1971; Tyson et al., 1971), decreased glucose and insulin, but elevated ketone acid levels in their blood (Felig and Lynch, 1970). It is believed that nutritional deprivation during fasting may be responsible for the elevation of the plasma PL concentration, and that an increase in hPL concentration during starvation may facilitate the mobilization and utilization of maternal FFAs for energy, thereby sparing maternal glucose for the fetus.

It has been suggested that, the main effects of oPL and hPL on maternal and fetal metabolism are increased gluconeogenesis and lipid mobilization, whereas bPL has no such obvious effects (Byatt et al 1992c; Handwerger and Freemark 2000). Earlier works with pregnant and non-pregnant cattle had shown that rbPL does not affect plasma concentrations of glucose, NEFAs or insulin, but increases plasma IGF-I and IGF-II to a lesser extent, as compared to bGH (Byatt et al., 1992c). Apparently, bPL displays different metabolic and hormonal changes in both pregnant and non-pregnant cows, as compared to bGH. This emphasizes bPL regulation of the maternal intermediary metabolism, perhaps through a specific receptor (Byatt et al., 1992c). Additionally, short-term (9d) treatment with rbPL induced an acute elevated feed intake in non-pregnant Holstein dairy cows (Byatt et al., 1992b). This effect was also obtained in sheep and cattle treated with bGH, but only after several weeks of continuous treatments (Bauman et al., 1985; Sandles et al., 1988). These data raise a possibility that bPL may regulate fetal growth via enhancing dry matter intake.

In summary, PL influences fetal growth by acting both on the fetus and the mother. These effects are exerted by altering maternal metabolism to ensure an adequate flow of substrates to the growing fetus, and by stimulating the fetus to use these substrates. This placental hormone may act through one or more IGF productions, and also through a structurally distinct PL-R, although the later theory remains controversial.

IMMUNOPURIFICATION OF BOVINE PLACENTAL LACTOGEN

Introduction

Placental lactogens (PL) have been isolated and purified in a number of various species, including human (Friesen, 1965), monkey (Shome & Friesen 1971), mouse (Colosi et al., 1982), rat (Robertson & Friesen 1975), sheep (Martal & Djiane 1975; Chan et al., 1976; Hurley et al., 1977; Reddy and Watkins 1978, Warren et al., 1990a) and goat (Currie 1990). The purification scheme for bovine placental lactogen (bPL) is more difficult than other placental proteins. Bovine PL was first isolated and purified from placental tissue by Bolander & Fellows (1976). This report demonstrated that the hormone was purified to approximately 99% homogeneity with an estimated molecular weight (MW) of 22,150 and its isoelectric point (pI) at 5.9. Isoelectric point is known as a pH at which a protein molecule bears no charge. This value varies between proteins. Additional purification studies were conducted in attempt to repeat the previous methods, but the bPL preparation yielded different characteristics. Murthy et al (1982) detected one form of bPL at 30,000 - 32,000 MW and 5.5 pI. Subsequent studies showed a 4,200-fold purification of bPL with several forms of bPL at 31,000 MW and several pI ranges from 5.39 to 5.85 (Arima and Bremel, 1983). Their purification procedures involved extraction and ammonium sulfate precipitation, followed by ultrafiltration, gel filtration, anion exchange, hydroxylapatite, chromatofocusing and final gel filtration chromatography. Wallace (1986) developed an efficient system for the purification of bPL from bovine fetal cotyledons using the initial purity of material from culture medium

thus eliminating the need for an ammonium sulfate precipitation step. Results showed that bPL was purified 616 fold and that two forms existed at 30,000 MW and two pI of 4.95 and 5.15. The existence of multiple forms of bPL with various pIs was identified from most previous works, using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) or chromatofocusing column.

In order to examine the structure and biological functions of PLs, there is a need for large quantities of purified molecules for additional *in vitro* and *in vivo* studies. Several laboratories have prepared recombinant bPL (Krivi et al., 1989), oPL (Colosi et al., 1989; Sakal et al., 1997), and cPL (Sakal et al., 1998) in sufficient amounts. Most of the recent studies, related to bPL, have utilized a recombinant molecule, which is structurally different from the native bPL (Byatt et al., 1992). The native molecule contains both O- and N-linked oligosaccharide in its structure and this makes it a higher MW molecule of 31,000 – 33,000 (Shimomura and Bremel 1988). There is no indication that PLs of sheep, goat and human are glycosylated. This explains why the MW of these PLs are lower (approximately 22,000) compared to that of bPL (Chan et al., 1976; Currie et al., 1990; Friesen, 1965). The enzymatic removal of N-linked sugar from native bPL increased the binding affinity of somatotropin receptor for bPL by about 1.2-2.3 fold. However, removal of O-linked oligosaccharide had a small effect on either somatogenic or lactogenic bindings (Byatt et al., 1990).

The purification of endogenous bPL, in quantities sufficient for complete physiological studies *in vivo* has not yet been reported. The low yield of native PL from bovine placenta may be one of the reasons for this limitation. An attempt has been made to clone the bPL cDNA into a mammalian expression vector, followed by an

amplification of the rbPL into *E. coli* cells and finally transfection of the recombinant molecule into mouse fibroblast cells (Doucette, 2003). It was expected that the mouse cells would be able to produce the bPL complete with the post-translational characteristics, which are observed in the native protein. The bPL cDNA was successfully cloned into the pcDNA 3.1 (+) vector, but the transfection process was not employed efficiently. The primary reason was that the cloned bPL gene was missing the third exon, which is known to be responsible for protein production.

Immunologic methods, such as affinity chromatography have proven to be a specific technique for the purification of biomolecules. One-step purification of nisin A, an antimicrobial peptide used in the food industry, by immunoaffinity chromatography has been reported (Suarez et al., 1997). The purification procedure resulted in 72% final yield and a 10-fold increase in specific activity. A similar method was accomplished to purify bPL from bovine fetal cotyledons, using the Affinica Antibody Orientation column, containing protein A agarose (Torto, 1994). It was hoped that this process would be more efficient than previously described methods in terms of rapidity, higher final yield and purity. The purification process was carried out starting from bovine fetal cotyledon homogenate, followed by an anion exchange chromatography and finally an Affinica column. The results showed that the Affinica column was not efficient to fully purify bPL, as a very poor final yield was achieved (3.6%).

Currently, immunomagnetic separation has been shown to be a very effective method for isolating various proteins, nucleic acids and cell types from a complex mixture. The principle of this technique involves coupling of an affinity ligand onto the magnetic particles. Beads are then directly added to the sample containing target

molecules. By doing this, the target has a chance to interact with an affinity group on the bead. Magnetic separation is achieved by placing the magnet outside a reaction flask. Thus the use of a centrifuge for concentration of molecules is not necessary. After washing the beads, the target molecule is eluted at low pH or in a high salt concentration. The application of a magnetic bead to separate a monoclonal antibody (Quitadamo and Schelling, 1998) and a human transferrin receptor (Karlsson and Platt, 1991) has proven to be more efficient than traditional affinity chromatography. One experiment was conducted to purify bPL from ammonium sulfate precipitate preparation, utilizing this novel approach (Miller, 1996). The purity of bPL at the end of purification was very low (10%). A further purification step was accomplished, using anion exchange chromatography (DE 52 diethylaminoethyl cellulose), but this chromatographic step did not provide adequate amounts and purity of bPL. Recombinant molecules, instead of the native protein, were used for further study in this experiment.

Since the previous procedures did not produce an efficient amount of purified protein from the bovine placental cotyledons, our challenge was therefore to establish a more rapid and specific method for the purification of bPL, using the immunomagnetic separation method. If a high yield and purity of the native molecule could be obtained, a comparative study of differences in biological activity between the native and recombinant proteins would be possible.

Materials and methods

Purification of antibody against bovine placental lactogen: Bovine placental lactogen was partially purified using the immunopurification method. The first step of bPL purification was to isolate an antibody (Ab) against bPL. This Ab was produced in sheep,

called sheep anti-rabbit Ab and was donated by Monsanto Corporation (Monsanto, St. Louis, MO). The Ab to bPL was purified using a 2 ml column of Immobilized Protein G (Pierce Biotechnology). The kit contained binding buffer (pH 5), elution buffer (pH 2.8) and immobilized protein G, which was isolated from group G streptococci. This type of protein binds to the mammalian IgG through their Fc regions and allows non-IgG components of the sample to pass on through the column. To facilitate binding, 2ml of serum sample was mixed with 2 ml of binding buffer. The sample was then loaded onto an equilibrated protein column and allowed to drain through the column. The column was washed by adding 10 ml binding buffer. The bound IgGs were recovered by adding 6 ml of elution buffer and immediately started to collect a 1.0 ml fraction into the first numbered collection tube. Column fractions were monitored for protein, using a spectrophotometer at 280 nm absorbance. The fractions having high absorbance, which contain the purified IgG, were combined. Concentration of IgG is described in $\text{mg/ml} = (A_{280} / 14) \times 10$.

Prior to introducing the purified Ab to the magnetic immobilization kit, the Ab activity was monitored by using a radioimmunoassay (RIA), then linked to an amine terminated biomag (Polysciences, Inc.). The coupling efficiency determined as the percent protein uptake was 79.8%, which met the requirement of manufacturer's recommendations (> 60%).

Purification of endogenous bPL: The ammonium sulfate (A.S.) precipitation, containing bPL from bovine placental cotyledons, was donated by Dr. Ron Kensinger (Pennsylvania State University). One gram of dried A.S. was dissolved in 40 ml Tris HCl buffer (10mM pH 7.5) and stored in the refrigerator overnight. Next day, the homogenate

was centrifuged at 10,000 x g for 30 min. The supernatant was collected and mixed with 5 ml biomag. This mixture was then loaded into a flask and rotated with low speed, at room temperature for 30 min. The solution was applied to the magnet for 15 min to pull bPL out of the solution. The liquid was drained off and saved for later analysis. The biomag was washed two times with Tris buffer (10mM pH 7.5) and the magnet was applied between washes. Removal of bPL from the biomag was done by adding 10 ml glycine (0.1 M pH 3.2). Finally, 3.33 ml 40% sucrose was added to the glycine mixture to prevent protein aggregation and then stored in a refrigerator for analysis.

At each step of the purification process, the protein content of the sample was estimated, measuring the absorbance of light at 280 nanometers with a spectrophotometer. The Bradford assay provided by Sigma was further used to re-examine the concentration of protein in the sample. This assay works by the action of the Brilliant Blue dye which specifically binding to the protein contained in the sample. This binding causes a shift in the absorption maximum of the dye from 465 to 595. The resultant increase in absorbance at 595 is recorded and the protein concentration is determined by comparison to a standard curve. A micro 2 ml assay protocol was utilized in our experiment following the instructions of the manufacturer.

The presence of bPL in the sample was detected by a double Ab RIA procedure, according to the method described by Wallace (1986). Each sample was run in duplicate and a standard curve with rbPL concentration ranging from .05 to 5 ng per tube was also constructed in duplicate. Recombinant bPL used for standards and for radioiodination was provided from Monsanto Corporation. The assay was run as follows: 100 μ l of sample or standard was combined with 200 μ l assay buffer (25 mM Tris-HCl, 50mM

EDTA, 0.1% BSA, pH 7.8), plus 200 μ l of the first Ab (rabbit anti-bPL IgG, diluted 1:15,000 in 1:400 normal rabbit serum in Tris buffer). The contents were mixed and incubated at 4°C for 24 h. The next day, each test tube was combined with 100 μ l of iodinated hormone (I^{125} bPL 20,000 cpm) and incubated at 4°C overnight. On the third day, antibody bound hormones were precipitated by addition of 200 μ l of the second Ab (sheep anti-rabbit IgG) and 500 μ l of 6% polyethylene glycol (PEG). Tubes were vortexed and incubated for 30 min at 4°C. Tubes were centrifuged at 1000 x g for 30 min and the supernatant was carefully decanted. Tubes were dried and the radioactivity of the precipitates was then counted in a Gamma Tract gamma counter. From the standard curve the unknown bPL in the sample could be determined.

Fractions that contained active materials were pooled and concentrated by lyophilization. The purity of the bPL was further confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Results and discussion

Bovine PL was purified from ammonium sulfate precipitate sample, using a novel immunomagnetic purification technique. In our experiment, 25 samplings were run following the procedures described above. The results of purifications are shown in Table 1 and Table 2 as follows.

Table 1: Immunomagnetic purification of bovine placental lactogen (bPL)

Sample	bPL (μg)	Protein (μg)	Purity: μg bPL / μg protein
bPL 1+2	1.44	-	-
bPL 3	7.15	49.0	0.15
bPL 4	3.60	35.0	0.10
bPL 5	38.40	152.0	0.25
bPL 6	7.44	121.5	0.06
bPL 7	13.29	107.8	0.12
bPL 8	12.60	74.2	0.17
bPL 9	18.00	60.9	0.30
bPL 10	22.80	84.0	0.27
bPL 11	11.40	64.0	0.18
bPL 12	7.08	72.0	0.10
bPL 13	10.80	88.2	0.12
bPL 14	5.88	74.3	0.08
bPL 15	5.52	27.0	0.20
bPL 16	2.52	40.0	0.06
bPL 17	28.80	180.0	0.16
bPL 18	12.00	103.2	0.12
bPL 19	12.00	127.2	0.09
bPL 20	0.38	164.0	0.002
bPL 21	0.84	170.5	0.005
bPL 22	-	179.2	-
bPL 23	36.0	176.8	0.20
bPL 24	15.0	69.6	0.22
bPL 25	25.2	21.0	1.20
Total	298.05	2241.4	0.13

Table 2: A summary of the purification procedure of bPL from ammonium sulfate precipitation

Procedure	Total bPL (μg)	Total protein (μg)	Purity $\mu\text{g bPL}/\mu\text{g protein}$
Homogenization	1226.80	168,000.00	0.007
Immunomagnetic purification	298.05	2241.40*	0.13
	298.05	1200.00**	0.25

(*): The 280 nm absorbance method

(**): The Bradford method

Initially, the purity of bPL was determined to be 13%, based on the value of the absorbance of light at 280 nanometers with a spectrophotometer to determine the protein content in the sample. We suspect that the purity of the sample preparation may be underestimated, as some colored components contained in the bPL preparation may interfere with the reading of the spectrophotometer, giving us a false estimate of protein. Further investigation was, therefore, carried out using the Bradford assay to re-examine the protein level in the sample. The purity level of bPL was re-estimated to be approximately 25%, which was higher than results obtained in the previous studies. In these experiments, the bPL molecule was purified starting from an ammonium sulfate precipitate sample, which is similar to that utilized in our experiment. The purity level of bPL was reported to be 9% and 10% by Torto (1994) and Miller (1996), respectively. Our level of purification, however, is still not high in comparison to some conventional purification approaches and some current studies using the magnetic beads to isolate various target molecules. The major advantage of our method is that it is simple and rapid.

Complicated devices required for the purification protocols are not necessary and only one-step purification is involved. At present, we have not investigated the exact cause of these limitations. We are considering that the use of a low pH eluting buffer (glycine HCl, pH 3.2) may have partially influenced the integrity of the immunoaffinity complex, which also may explain the low yield and purity observed in our study. There is evidence to suggest that the optimal elution buffer used in the magnetic bead separation has increased the yield and purity of the targeted molecules, minimizing the dissociation of protein. Recent investigations demonstrated the physical degradation of magnetic beads when using the low pH eluting buffer in their experiments, including urea 6 M-formic acid 0.1M, pH 3.5 and glycine HCL pH 2.5 (Prioult et al., 2000). The efficiency of using a magnetic bead purification method for isolating mouse (Quitadamo & Schelling, 1998) and rabbit (Quitamo et al., 2000) monoclonal antibodies has been reported. The use of extreme pH in these studies has been avoided to maximize the recovery of immunoreactive proteins. The earlier data achieved 70% for magnetic bead purification efficiency, using high salt instead of low pH for antibody elution. The latter report indicated the rabbit IgG was eluted from magnetic beads with acetic acid 0.5M, pH 4.5 to avoid harsh condition. The average percent of IgG yield estimated was 78%.

Furthermore, different sizes of magnetic beads, used in the purification process also have been reported to influence the recovery and specificity of the target molecules. The advantage of using micromagnetic beads (diameter < 50nm) over the large beads (> 500 nm) in detecting bacterial cells has been demonstrated (Jacobsen et al., 1997; Miltenyi et al., 1990). Results showed the recovery of bacterial cells up to 95% by using the micromagnetic bead without the aggregation of beads (Jacobsen et al., 1997).

In summary, immunomagnetic separation was used to purify bPL from an ammonium sulfate precipitation sample, yielding a purity level of 25%. The SDS-PAGE analysis showed the presence of a band about 32,000 MW, however, some contaminants appeared on the gel thus further isolation steps are needed. It is not clear what is the primary cause influencing the results, although some factors such as elution buffer and the size of the magnetic beads have been noted to affect the purity and final yield of various proteins or cells. In general, it is difficult to compare purification processes that involve different target proteins. Purification schemes for bPL may require some optimal conditions as indicated above, but to elucidate this needs further investigation.

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